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A direct proofreader-clamp interaction stabilizes the Pol III replicase in the polymerization mode

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19 November 2012

Thank you for submitting your manuscript direct proofreader-clamp interactions in DNA polymerase III for consideration by The EMBO Journal. Three expert referees have now assessed it, and I am pleased to inform you that all of them consider this work of interest and appropriate for publication, pending adequate revision of a number of specific points. I would therefore like to invite you prepare a revised version of the manuscript, addressing the various textual and experimental points raised by the referees, for our further consideration. When preparing your letter of response, please be reminded that our policy to allow only a single round of revision will necessitate diligent and comprehensive answering, and also bear in mind that this letter will form part of the Peer Review Process File available online to our readers in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html).

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published here or elsewhere during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work for publication. I look forward to your revision.

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Many of the strong interactions that stabilize the replicative DNA polymerase III holoenzyme (Pol III HE) that are maintained throughout the process of replication have been identified whereas weaker interactions remain poorly defined due to inherent difficulties associated with their identification. By utilizing in vitro replication, SPR, mass spec, and single molecule experiments, Jergic et al define a weak, but functionally important, interaction between the epsilon exonuclease subunit and the beta processivity clamp from the Pol III HE. This weak interaction plays a role in regulating access of alternative polymerases to the beta clamp and to the replication fork. This paper beautifully integrates a diverse array of methods and data to generate a well supported and novel physical model explaining the arrangement of subunits within the polymerase core/clamp complex. Only minor points are raised below for the authors to consider.

1. Figure 3A. The authors generate a possible fit for the epsilon(Q) mutant peptide from only two point but this is a bit misleading since they rightly report a lower limit of ~2 mM for the K(d) in the paper. This fit line should be removed from the figure.

2. Figure 6. The authors should include error estimates of the calculated values for processivity, rate and lifetime in the single molecule experiments.

3. There were a small number of typographical errors in the text.

Referee #2 (Remarks to the Author):

Summary: The manuscript by Jergic et al. takes advantage of a strand displacement (SD) DNA synthesis activity of the E. coli DNA polymerase III (Pol III) holoenzyme complex originally reported by the McHenry lab, surface plasmon resonance (SPR), electrospray-ionization mass spectroscopy (ESI-MS), and a single molecule Pol III replication assay to uncover and characterize an interaction involving the Pol III epsilon proofreading and the Pol III sliding processivity clamp subunits. Evidence supporting the model that this interaction contributes to the stability of the Pol III replicase is presented, and is well discussed. This reviewer is not an expert in ESI-MS, and does not feel fully qualified to expertly review this aspect of the work. With this said, this is an overall compelling story that provides novel insights into the structure and dynamics of the E. coli replicase, and offers important clues into how the polymerization and exonuclease proofreading functions are coordinately managed. Two suggestions regarding the presentation are made below; neither is intended to detract from what is already an outstanding presentation.

Specific comments:
1. The author's present several results using the SD DNA synthesis assay. Currently, these data are presented in a qualitative fashion, but the data could be made quantitative by merely measuring levels of the different products (i.e., TFII, SD, unreplicated M13 template). This presentation would facilitate analysis by the reader, and would also provide more weight to the author's arguments for effects of epsilon and the V832G mutation in Pol III alpha.

2. I would encourage the authors to provide additional details regarding their view on the impact of the Pol III epsilon-clamp interaction with regard to models for Pol III-Pol IV switching involving either both clefts as proposed by Indiani and colleagues in Mol. Cell, [2005] 19, pp 805-815, or a single cleft, as proposed by Heltzel and colleagues 2009 Proc. Natl. Acad. Sci. USA, [2009] 106, pp 12664-12669. Based on their current discussion, it is not clear to this reviewer how the epsilon-clamp interaction influences the published models.

Referee #3 (Remarks to the Author):

S. Jergic et al. set out to identify new protein-protein interactions in the E. coli DNA polymerase III

replicase using a strategy that required the replicase to synthesize DNA under challenging strand-displacement conditions. This creative approach lead to the discovery of a novel interaction between the epsilon proofreading subunit of DNA polymerase III and the beta clamp. Further detailed characterization of the requirement of epsilon carryout strand displacement synthesis showed that it was not dependent on the proofreading activity of epsilon but instead uncovered a new function for epsilon in stabilizing the replisome. Previous work from this group and others had identified two clamp binding motifs (CBM) within the alpha polymerase subunit of DNA polymerase III, and this study uncovered a CBM in epsilon. Overall, this is a great piece of work that used many different approaches to support their conclusion that the epsilon contains a CBM that is important for stabilizing the replicase and for processivity under DNA synthesis conditions.

Specific questions:

1) It is not clear why the authors deleted one of the CBM's in alpha but strengthened the other. Strengthening the internal CBM could cause it to be used in a manner that it is not normally used under wild-type conditions. Why wasn't the internal CBM mutated to make it weaker like the terminal CBM so that similar loss of function mutations could be compared?

2) Is the lower right panel in Figure 6 "Half-life" as in panel F or "Lifetime" as labeled?

3) Panel 7B seems a bit speculative. Can the authors exclude the possibility that the "closed" state in which both the alpha and epsilon subunits are bound isn't a proofreading state? And that when the polymerase is incorporating nucleotides both CBM's in alpha bind beta, but when it switches to proofread (even in the proofreading mutants) alpha and epsilon bind beta by their CBM's. Is it possible that destabilizing any one of the three CBM's weakens the interaction such that the complex becomes destabilized during the process of switching from polymerase to proofreading activities?

1st Revision - authors' response 27 November 2012

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Jergic et al: A direct proofreader–clamp interaction stabilizes the Pol III replicase in the polymerization mode

RESPONSE TO REFEREES' COMMENTS

The authors are very grateful for the thought-provoking and very helpful comments of all three referees.

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Many of the strong interactions that stabilize the replicative DNA polymerase III holoenzyme (Pol III HE) that are maintained throughout the process of replication have been identified whereas weaker interactions remain poorly defined due to inherent difficulties associated with their identification. By utilizing in vitro replication, SPR, mass spec, and single molecule experiments, Jergic et al define a weak, but functionally important, interaction between the epsilon exonuclease subunit and the beta processivity clamp from the Pol III HE. This weak interaction plays a role in regulating access of alternative polymerases to the beta clamp and to the replication fork. This paper beautifully integrates a diverse array of methods and data to generate a well supported and novel physical model explaining the arrangement of subunits within the polymerase core/clamp complex. Only minor points are raised below for the authors to consider.

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This line has been removed from the figure and the K_D > 2 mM noted in the legend (derivation of this estimate is described in the legend of Supplementary Figure S3).

2. Figure 6. The authors should include error estimates of the calculated values for processivity, rate and lifetime in the single molecule experiments.

These error estimates were already given explicitly in the earlier panels of Figure 6 (for the wild-type Pol III core) and Supplementary Figure S8 (for α alone and the mutant cores), and were shown graphically in Figure 6G. We have now also explicitly included the estimates and standard errors from fitting in Figure 6G.

3. There were a small number of typographical errors in the text.

We have carefully rechecked the text and corrected typographical errors.

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Referee #2 (Remarks to the Author):

Summary: The manuscript by Jergic et al. takes advantage of a strand displacement (SD) DNA synthesis activity of the E. coli DNA polymerase III (Pol III) holoenzyme complex originally

reported by the McHenry lab, surface plasmon resonance (SPR), electrospray-ionization mass spectroscopy (ESI-MS), and a single molecule Pol III replication assay to uncover and characterize an interaction involving the Pol III epsilon proofreading and the Pol III sliding processivity clamp subunits. Evidence supporting the model that this interaction contributes to the stability of the Pol III replicase is presented, and is well discussed. This reviewer is not an expert in ESI-MS, and does not feel fully qualified to expertly review this aspect of the work. With this said, this is an overall compelling story that provides novel insights into the structure and dynamics of the E. coli replicase, and offers important clues into how the polymerization and exonuclease proofreading functions are coordinately managed. Two suggestions regarding the presentation are made below; neither is intended to detract from what is already an outstanding presentation.

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1. The author's present several results using the SD DNA synthesis assay. Currently, these data are presented in a qualitative fashion, but the data could be made quantitative by merely measuring levels of the different products (i.e., TFII, SD, unreplicated M13 template). This presentation would facilitate analysis by the reader, and would also provide more weight to the author's arguments for effects of epsilon and the V832G mutation in Pol III alpha.

We believe the referee has missed that in Figures 2 and 5 we are presenting negative images of photographs of gels that had been stained with SYBR gold, a stain that fluoresces when bound to either single- and double-stranded DNA, rather than using phosphorimaging of ^{32}P-labeled DNA products. Photography at this resolution has a much more limited dynamic range than phosphorimaging, and it is also difficult to quantify the relative amounts of single- and double-stranded DNA from densitometry when we do not know their relative staining efficiencies. Although we attempted to analyze images as suggested, and obtained data consistent with the qualitative data, we believe it would be an over-interpretation of our results to pretend that we can reliably extract quantitative data. Our images have not been manipulated in any way except to convert them to negatives to aid visual presentation (and print production). We have added the sentence to the legends of these Figures for clarity: "All panels show photographic negative images of gels that had been stained with SYBR® gold nucleic acid stain (Invitrogen)".

2. I would encourage the authors to provide additional details regarding their view on the impact of the Pol III epsilon-clamp interaction with regard to models for Pol III-Pol IV switching involving either both clefts as proposed by Indiani and colleagues in Mol. Cell, [2005] 19, pp 805-815, or a single cleft, as proposed by Heltzel and colleagues 2009 Proc. Natl. Acad. Sci. USA, [2009] 106, pp 12664-12669. Based on their current discussion, it is not clear to this reviewer how the epsilon-clamp interaction influences the published models.

We have rearranged and slightly expanded the Discussion on pages 19–20 to address this issue. The text now reads:

"Our data strongly suggest that the two sites in the β dimer are occupied simultaneously by ε and the internal CBM of α during processive DNA replication. There are at least two situations where synthesis by α might stall to signal a conformational switch to break just the ε–β contact without the polymerase dissociating from the DNA template, or at least change the location of the ε active site in the replicase complex. These are (i) during lesion bypass or repair synthesis by the alternate polymerases Pol II, IV or V (Indiani *et al*, 2005; Furukohri *et al*, 2008; Heltzel *et al*, 2009), and (ii) during proofreading. It has been suggested that in PolC (Evans *et al*, 2008), entry of alternate polymerases occurs *via* transition from the closed primer-template-bound

structure (similar to the model in Figure 7A) to an open one reminiscent of α in the absence of DNA (Figure 7B). This transition would require that the ε–β contact be broken, providing access of the CBM of the incoming polymerase to β_2.

"There are two separate models for how primer-template DNA is switched from α to an alternate polymerase (reviewed by Sutton, 2010). In the toolbelt model (Pagès and Fuchs, 2002; Indiani *et al*, 2005; López de Saro *et* al, 2003a), a repair or lesion-bypass polymerase would trap the replicase in the open state by temporarily replacing ε at its binding site in β_2 to access the primer terminus while α remains attached at the other; ε would remain tethered to α through a flexible linker (Ozawa *et al*, 2008), enabling it to re-establish contact with β_2 when processive synthesis by α is resumed. The second model, demonstrated with Pol IV, involves switching of polymerases at the same protein binding site on the β_2 ring (Heltzel *et al*, 2009), and requires a secondary contact between Pol IV and β. Evidence for this model is that Pol III/IV switching can still occur efficiently on a β^C/β_{wt} heterodimer that has only one functional protein-binding site. In this more desperate situation, the Pol III core is apparently able to disengage from β_2 while still remaining in the replicase through its contacts with τ. This may not be an unusual situation, since exchange of τ-bound Pol III cores between different clamps certainly occurs during their recycling to new primer termini on completion of lagging strand Okazaki fragment synthesis. In this process, contacts of both α and ε with β_2 must be broken. The differences, if any, among replicase stalling signals in these various situations and how they affect transactions of CBMs on the sliding clamp is an area where we still have much to learn.

"Proofreading also involves replicase stalling and requires transfer of a mismatched primer-template from the active site of α to that of ε. It has been suggested (Evans *et al*, 2008; Wing *et al*, 2008) that this might also require at least partial opening of the closed DNA-bound structure (as in Figure 7A) to pull the primer-template from the polymerase site to access the exonuclease site of ε. There is some evidence for this opening. The Pol III replicase can be stalled in a stable complex at a primer terminus when only two of the four dNTPs are present, where it undergoes futile cycles of nucleotide misincorporation and proofreading. In this situation, it has been found to be more prone to exchange with an alternate polymerase than when it is actively replicating DNA (Indiani *et al*, 2005; Furukohri *et al*, 2008; Heltzel *et al*, 2009). This would be nicely explained if breakage of the ε–β contact occurred during proofreading to allow easier access of the incoming polymerase."

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Referee #3 (Remarks to the Author):

S. Jergic et al. set out to identify new protein-protein interactions in the E. coli DNA polymerase III replicase using a strategy that required the replicase to synthesize DNA under challenging strand-displacement conditions. This creative approach lead to the discovery of a novel interaction between the epsilon proofreading subunit of DNA polymerase III and the beta clamp. Further detailed characterization of the requirement of epsilon carryout strand displacement synthesis showed that it was not dependent on the proofreading activity of epsilon but instead uncovered a new function for epsilon in stabilizing the replisome. Previous work from this group and others had identified two clamp binding motifs (CBM) within the alpha polymerase subunit of DNA polymerase III, and this study uncovered a CBM in epsilon. Overall, this is a great piece of work that used many different approaches to support their conclusion that the epsilon contains a CBM that is important for stabilizing the replicase and for processivity under DNA synthesis conditions.

Specific questions:

1) It is not clear why the authors deleted one of the CBM's in alpha but strengthened the other. Strengthening the internal CBM could cause it to be used in a manner that it is not normally used under wild-type conditions. Why wasn't the internal CBM mutated to make it weaker like the terminal CBM so that similar loss of function mutations could be compared?

Excellent work by Dohrmann and McHenry (as cited, 2005) clearly showed already that only an internal CBM in α is required for processive replication by *E. coli* Pol III HE. Indeed, they showed that replacement of the internal CBM (QADMF) in α by AAAKK not only eliminated detectable binding to β_2 (affinity for β_2 was reduced >130-fold compared to wild-type α), but rendered α incapable of DNA replication *in vitro* and *in vivo*, while at the same time, it did not affect its intrinsic polymerase activity (without β_2). Intriguingly, the complete removal of the C-terminal CBM ($\alpha\Delta 7$) reduced binding to β_2 only 2-fold and did not have serious effects on DNA replication *in vitro*. We think that these results suggest that the external and comparably strong (Wijffels *et al*, 2004; López de Saro *et al*, 2003b) C-terminal CBM in α (QVELEF) may not normally be fully accessible to β_2; it is known to be close to or part of the τ-binding site in α.

Given this background and the focus of this present manuscript on the discovery and significance of the ε–β interaction, we initially sought to establish conditions to detect this interaction in the context of the $\alpha\varepsilon\theta$–β_2 complex by ESI-MS. Accordingly, we first selected those mutants of α whose interaction with β_2 could be expected to be detectable by this technique, for comparison with complexes formed in the presence of ε (and its mutant versions). We consider that the range of α mutants we used in our work (MS and *in vitro* replication assays) provided proof that β_2 in the (DNA-free) $\alpha\varepsilon\theta$–β_2 complex interacts with the CBM in ε and the internal CBM in α, and is entirely in accord with Dohrmann and McHenry (2005) with respect to the roles of the CBMs in α. Having said that, we agree with the suggestion that MS measurements with α that has the internal CBM weakened might have been informative. Indeed, we were ready to explore that path had our measurements (MS, replication assays) not yielded convincing results consistent with previous work.

2) Is the lower right panel in Figure 6 "Half-life" as in panel F or "Lifetime" as labeled?

This oversight has been corrected, both in the Figure 6 and the text, to avoid confusion given that both “lifetime” and “half-life” of first-order kinetic processes have precise definitions.

3) Panel 7B seems a bit speculative. Can the authors exclude the possibility that the "closed" state in which both the alpha and epsilon subunits are bound isn't a proofreading state? And that when the polymerase is incorporating nucleotides both CBM's in alpha bind beta, but when it switches to proofread (even in the proofreading mutants) alpha and epsilon bind beta by their CBM's. Is it possible that destabilizing any one of the three CBM's weakens the interaction such that the complex becomes destabilized during the process of switching from polymerase to proofreading activities?

We have some confidence in the models used for illustrative purposes in Figure 7. They are based on a series of experimental observations and reasonable modeling (as described in detail in the cited references to Wing *et al*, 2008 for the closely-related *Taq* α and Evans *et a*l, 2008 for the more distantly related *Geobacillus kaustophilus* PolC): (1) The crystal structures of *E. coli* α(1–917) (Lamers *et al*, 2006) and full-length *Taq* α (Bailey *et al*, 2006) in the open state are

essentially superimposable in the region that can be compared, and the organization and sequences of the remaining domains are similar enough that they would be expected to be in similar positions (notwithstanding the fact that *Taq* α and α from most other bacteria do not have a recognizable C-terminal CBM). (2) The closed structure of *Taq* α with primer-template and incoming dNTP (and therefore in the polymerization mode as modeled in Figure 7A) shows a 20˚ rotation of the clamp-binding (fingers) domains and a smaller movement of the N-terminal PHP domain inwards towards the dsDNA (Wing *et al*, 2008). (3) The structure of the β_2–DNA complex (Georgescu *et al*, 2008b) can be almost seamlessly docked onto the primer-template DNA in the closed *Taq* α–DNA structure (as described by Wing *et al*, 2008) so that the highly-conserved internal CBM of α fits neatly into one of the protein-binding pockets of β_2. This therefore positions the other binding pocket in β_2 towards the N-terminal PHP domain of α, far from the predicted position of the C-terminal CBM in *E. coli* α. Note that we have now included the position of C-terminus of α in the model in Figure 7A to illustrate this. (4) The exonuclease domain in PolC is incorporated into the PHP domain (but was removed from it for crystal structure determination) while in *E. coli* the same domain is connected to the N-terminal PHP domain (Wieczorek and McHenry, 2006) *via* a flexible linker (Ozawa *et al*, 2008) enabling it (in its complex of known structure with θ) potentially to sample a relatively large space in any of the conformational states. (5) Our present work establishes a new point of contact between the CBM in ε and the second protein-binding cleft in β_2, and this provides an additional restraint that allows us to sandwich εθ between the PHP domain of α and the second binding site in β_2, but not to define its precise position (as shown in Figure 7A, which due to the structures on which it is based must represent the closed complex in the polymerization mode).

The illustrative model in Figure 7B is indeed more speculative, but we note that we have been careful in our discussion not to ascribe it confidently to either the conformational state present during proofreading or during entry of an additional clamp-binding protein or polymerase, nor to attempt to position primer-template DNA in it. It is constructed on the reasonable assumption that the local structure in the region of the contact between the conserved internal CBM of α and β_2 is maintained in the open state (see below), and that the "open" crystal structures obtained with α subunits from two different species probably have some biological significance. If one visualizes how the primer-template in this model might be placed (through the center of the clamp), it is easy to imagine that it could now be readily accessed by a lesion-bypass polymerase or by the exonuclease active site of ε. We consider it possible (even probable) that during proofreading the replicase would need to open less far than this, but that is a topic for future work.

We believe that in the polymerization and proofreading modes, and probably also during the simplest forms of polymerase exchange (see above), the more poorly-conserved C-terminal CBM in α is occluded by interaction with the C-terminal domain of τ (Dohrmann and McHenry, 2005; Jergic *et al*, 2007), and is not therefore relevant to construction of these models.

With respect to the very last question, we cannot speculate on how weakening of either of the two CBMs in α would affect the stability of the replicase as it transits between polymerization and proofreading modes. Certainly, our single-molecule experiments show that weakening of (or even completely removing) the ε–β contact does not affect the overall stability (as measured by the half-lives in Figure 6G). We have been careful, however, not to over-interpret this since we really don't know how much time the replicase spends in the proofreading mode during DNA synthesis under these conditions. Similar measurements with appropriate mutant proteins under conditions where proofreading becomes significant could in the future help to answer this question.

Acceptance letter 07 December 2012

Thank you for submitting your revised manuscript for our consideration. After a brief delay due to travel-related absence from the office, I have now had a chance to look through the new manuscript and your responses to the original three sets of reviewer comments. I am pleased to inform you that in light of these satisfactory modifications and responses, we have decided to accept your manuscript for publication in the EMBO Journal!

Thank you again for your contribution to our journal, and congratulations on a successful publication. We hope you consider us again for your most exciting work in the future.

